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Natural Sources and Properties of Chemical Inducers Mediating Settlement of Oyster Larvae: A Re-examination

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Abstract. Live adult oysters and biofilms were separated experimentally as potential sources of waterborne chemical inducers of settlement in oyster larvae (*Crassostrea virginica*). Bacteria films growing on external shell surfaces were removed by mechanical agitation and chemical oxidation. This technique removed >99% of the viable bacteria without disrupting the normal production of metabolites by the oysters, measured as the weight-specific production of ammonium and dissolved organic carbon (DOC). In comparison to the external biofilms, microfloral abundances in oyster tissues and on internal shell surfaces were numerically insignificant (≤0.1% of total). Biofilms growing on aged shell material without the living oyster served as a source of bacteria metabolites. Metabolites released in particle-free, artificial seawater (ASW) medium by biofilms and by adult oysters (lacking biofilms) were tested for effects on larval behavior, relative to ASW (control). The larvae were exposed to solutions in a Plexiglas® microcosm (30 ml capacity). Locomotory responses were video recorded under infrared illumination, then subjected to computer-video motion analysis. Oyster larvae responded similarly to waterborne substances released *both* from adult conspecifics and from biofilms. The responses included: larvae rapidly swimming vertically downward in the water column; their horizontal swimming speed then slowed while their rate of turning increased, which focused activity near the bottom; and finally, the larvae contacted the bottom and attached with their foot, indicating settlement. Further analysis demonstrates that the settlement-inducing compounds of each source have a molecular weight between 500 and 1000.

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Introduction

Adults of most marine invertebrates reproduce sexually, by shedding gametes into the surrounding seawater where external fertilization occurs (Simpson and Beck, 1965). The embryos then develop into larvae which may spend hours to months in the water column before metamorphosing into the juvenile form. Larvae are often suspended and carried by ocean currents that serve as the agents of dispersal for parental stocks (Quayle, 1969; Roff, 1974; Hamilton and May, 1977; Palmer and Strathmann, 1981; Levin, 1984). Larval dispersal is especially important for species with sessile or sedentary adults. A vast majority of sessile marine animals, such as barnacles, oysters, tube-dwelling worms, and bryozoans produce planktonic larvae.

Colonization of benthic environments requires that larvae both settle and metamorphose. Settlement is a reversible behavioral process which includes the contact and exploration of substrates by larvae before metamorphosis. Metamorphosis is an irreversible developmental process mediating the biochemical, physiological, and morphological transformation of an individual between distinct life forms. Settlement and metamorphosis can be distinct processes and, in some cases, appear to differ in their regulatory pathways (Bonar *et al.*, 1990; Coon *et al.*, 1990a),

although the compounds that induce settlement and metamorphosis are usually identical for larvae of a given species (*e.g.*, reviewed in Morse, 1990). In any event, mechanisms of both settlement and metamorphosis must be elucidated before the processes controlling habitat colonization by larvae can be fully understood.

Gregarious settlement by larvae can lead to aggregations of conspecific adults (Meadows and Campbell, 1972; Burke, 1986; Gotelli, 1990). Aggregation resulting from larval settlement may be critical when the adult form is sessile or sedentary and reproduces sexually by spawning gametes into surrounding waters. In these cases, the hydrodynamical properties of turbulent-flowing water tend to dilute and disperse gametes after release. This dilution decreases the likelihood of fusion between egg and sperm, a consequence similar to that of increasing distance between spawning individuals of opposite sexes (Pennington, 1985; Grosberg, 1987; Denny and Shibata, 1989; Levitan, 1991). In addition to improving reproductive success (Knight-Jones and Stevenson, 1950; Crisp, 1979), gregarious settlement that leads to aggregation among juvenile and adult conspecifics may increase protection from predation (Sebens, 1983; Keough, 1984), competitive ability (Buss, 1981), and filter-feeding efficiency (Hughes, 1978), while also reducing juvenile and adult mortality (Knight-Jones, 1951; Buss, 1979; Highsmith, 1982; Young, 1983). Since gregariousness is critical for many sessile and sedentary species, specific traits that promote the active selection of settlement sites by larvae should be expected.

Gregarious settlement is believed to occur in response to chemical cues emitted by adult conspecifics (see reviews by Crisp, 1974; Burke, 1986; Pawlik and Hadfield, 1990). Considerable progress in identifying chemical cues has been made in some cases (*e.g.*, Pawlik and Faulkner, 1986; Jensen and Morse, 1990), although substances mediating gregarious settlement have yet to be completely isolated and fully characterized. Since Cole and Knight-Jones (1939) first described gregarious larval settlement and metamorphosis in oysters, there has been debate about the sources of settlement-inducing compounds. Two schools of thought have emerged: one points to juvenile and adult oysters (Walne, 1966; Bayne, 1969; Hidu, 1969; Keck *et al.*, 1971; Veitch and Hidu, 1971; Hidu *et al.*, 1978); whereas the other finds that biofilms on oyster shell surfaces (Bonar *et al.*, 1986, Fitt *et al.*, 1989, 1990; Weiner *et al.*, 1989) are the source of inducer molecules. These contrasting viewpoints may result from differences in experimental approach. Significantly, the effects of substances released by live adult oysters and by biofilms have never been separately assayed in a single study.

We have now quantified larval responses to waterborne substances released by each source. Our results show that: (1) settlement inducers are produced both by oysters and biofilms, (2) larval settlement behavior in response to inducers from each source is essentially identical, and (3) the settlement inducers liberated by both sources have a molecular weight between 500 and 1000.

Materials and Methods

Larval cultures

Twelve day old larvae, raised from Chesapeake Bay oysters and spawned at the Virginia Institute of Marine Science, were shipped via overnight courier to our laboratory. They were maintained at 0.5 to 1.0 larvae/ml in a 1:1 mixture of oceanic and artificial seawater at 25 ppt salinity, pH 8.0, in a 25°C incubator with a 12:12 dark: light cycle (light on: 0700 h). Prior to use, the culture media was filtered to 0.22 μm and autoclaved for 15 min at 150°C and 15 psi. Cultures were aerated actively by air bubbled through Pasteur pipettes. The culture medium was changed daily to preclude the build-up of pathogenic bacteria (Loosanoff and Davis, 1963), and marine diatoms (*Isochrysis galbana* and *Pavlova lutheri*) were provided as food, at 2.5×10^4 cells/ml, once each day.

Oyster larvae are typically >250 μm in length and have pigmented eye spots when competent to settle and metamorphose (Galtsoff, 1964; Coon *et al.*, 1990a). Experiments were begun within 6 h after 100% of the larvae in a culture had developed eyes, and experiments were run for 24 h thereafter. The mean length of larvae tested was 310 μm ($\pm$11 μm SD).

Summary of experimental procedure

Due to the complexity of the protocol, the following brief overview is provided so that the various specific methods outlined later can be kept in perspective. Nine solutions (including controls) were prepared to isolate substances released by adult oysters, biofilms, and empty oyster shells (called 'cultch'). The ammonium and dissolved organic carbon (DOC) in each solution were measured as an indication of general metabolite levels.

Methods were developed for removing the bacterial films covering the outer surface of adult oysters and shell cultch. We focused on eliminating external biofilms because the microflora inside of oysters was numerically insignificant in comparison. The total number of bacteria in oyster tissues, on inner shell surfaces, and released into bath solutions during incubations of oysters were measured, and found to be $\leq$0.1% of the total on the external shell surfaces. The internal microflora are therefore not discussed further in this report; these data are available from M. N. Tamburri on request.

Oysters with their external biofilms removed served principally as a source of oyster metabolites. Biofilms growing either on live oysters that were clamped shut, or

Table I

Schematic representation of methods used to treat live oysters during the preparation of bath-water solutions for experiment I

Live oysters	Treatment: Clamps	Treatment: Scrub and chemical oxidation	Sources of dissolved compounds	Abbreviation
Test solutions				
(+)	(−)	(−)	Oyster + Biofilm + Shell	OBS
		(+)	Oyster + Shell	OS
	(+)	(−)	Biofilm + Shell	BS1
		(+)	Shell	S1
Control solutions				
(−)	(+)	(−)	Clamp	CC
	(−)	(−)	None	SWC1

(+) Indicates the presence of oysters or use of treatment; (−) indicates the absence of oysters or omission of treatment.

on aged shell cultch, served as a source of biofilm metabolites. The oysters were shut with C-clamps wrapped in sterile polyethylene. Epifluorescence microscopy, Most Probable Number (MPN) analysis, and direct plate counts were used to enumerate the bacterial densities found in biofilms, and to estimate the effectiveness of our biofilm removal technique.

The locomotory responses of larvae to test and control solutions were subjected to computer-video motion analysis. Settlement behavior was characterized as: downward movement, slowed swimming speed with increased horizontal turning near the bottom, and finally, contact with and attachment to the bottom. The solutions that induced oyster larvae to settle were serially diluted so that the relationship between inducer dose and larval behavior could be determined. In addition, solutions causing settlement of larvae were fractionated by molecular weight as a first step in the characterization and identification of the inducers. Specific methods used in our study are described in detail below.

Preparation of solutions

Live oysters and shell cultch were collected from reefs in Portersville Bay, Alabama, in May, 1990. These materials were transported to our laboratory at Dauphin Island, Alabama, in aerated seawater in polyethylene containers. The methods used in preparing solutions are shown schematically in Tables I and II. Test solutions were made by bathing either six oysters or six shells, for 4 h, in 4 l of sterile ASW held at the same temperature (27°C), salinity (17 ppt), pH (7.8), and dissolved oxygen (6.8 mg/l) as seawater at the collection site. Each group of oysters had a total wet tissue weight of 200 ± 18 gm, and a total shell surface area of 700–800 cm^2. After the 4 h bath, we removed the oysters or shells and adjusted the pH to 8.0 with 1 *M* NaOH, and the salinity to 25 ppt; these conditions are the same as those of the larval culture medium. The solutions were filtered to 0.22 μm, divided into 25 ml aliquots, and frozen at −87°C until used in experiments. All lab wares were sterilized before use, either by autoclaving (15 psi, 150°C, 15 min) or, when auto-

Table II

Schematic representation of methods used to treat oyster shell cultch during the preparation of bath-water solutions for experiment II

Shell cultch	Treatment: Scrub and chemical oxidation	Sources of dissolved compounds	Abbreviation
Test solutions			
(+)	(−)	Biofilm + Shell	BS2
	(+)	Shell	S2
Control solutions			
(−)	(−)	None	SWC1

(+) Indicates the presence of cultch or use of treatment; (−) indicates the absence of cultch or omission of treatment.

claving was impossible by rinsing with 70% isopropyl alcohol.

Our first experiment tested the capacity of solutions, prepared with adult oysters, to induce settlement of oyster larvae (Table I). Our second experiment tested the larval response to solutions containing compounds released only by biofilms and oyster shell material (Table II), and thus enlarged on the results of the first experiment.

Larval behavioral assays

Two to three hours before testing, the larvae were gently filtered from the culture media, rinsed, then placed at 0.5 larvae/ml in a separate container of ASW (25 ppt). This procedure removed the oyster larvae from their microalgal food, the exudates of which are known to influence the locomotory behavior (Zimmer-Faust and Tamburri, pers. obs.). Sixty larvae (±2) were then transferred, in 5 ml ASW, into a Plexiglas® microcosm (3 cm long × 3 cm wide × 4 cm high) containing 25 ml of a test or control solution. The solution containing the larvae was gently stirred for 5 s, then held briefly until the fluid came to rest. The microcosm was next placed into a darkened chamber at 25°C, where the larval movements were recorded on video tape. The chamber was illuminated with an infrared light source (>820 nm) oriented 90° to the axis of the video field. The use of infrared light was required because oyster larvae exhibit phototactic responses to visible wavelengths (Smith and Chanley, 1975). The larvae were taped with a Sony infrared-sensitive video camera (Model HVM-200) equipped with a Tamron 180 mm macrofocal lens. Video records made with a Panasonic (Model AG-6300) video cassette recorder, were stored on magnetic tape, and viewed with a Panasonic (Model TR-124MA) monitor.

During the experiments, we recorded the paths made by larvae as they: (1) swam vertically in the water column, and (2) swam horizontally near, then contacted the bottom. Eight replicate trials were run for each solution and recording position. Behavior was monitored during the initial 3 min of exposure, corresponding to the period of the maximum larval settlement response (Zimmer-Faust and Tamburri, pers. obs.). The order of test and control solutions was determined with a random numbers table, subject to the constraints that: each solution was presented in every block of nine trials; and no single solution was ever tested twice in succession. Individual larvae were tested only once, then discarded.

Vertical swimming in the water column. The video camera was mounted at the side of the microcosm to observe larvae swimming vertically, 1.5 cm (40–50 body lengths) above the bottom. The size of the viewing field was 6.9 mm × 6.8 mm, with a 1.5 mm depth. We programmed a computer-video motion analyzer to count the larvae moving vertically upward and downward across the viewing field. The number of larvae swimming downward was subtracted from the number swimming upward through the plane per unit time, giving a quantitative measure of net vertical flux. A negative value is net downward movement, while a positive value is net upward movement.

Horizontal swimming near, and settlement on bottom. The camera was mounted beneath the microcosm to view larvae as they swam in a horizontal plane (6.75 mm × 6.70 mm) at a distance, ≤1.2 mm (≈4 body lengths) above the bottom. Swimming paths were recorded and then subjected to computer-video motion analysis. We counted only those larvae that attached with the foot to the bottom during the observation period as a measure of settlement.

Computer-video motion analysis of larval responses to test and control solutions. We quantified swimming speed and turning behavior by replaying each video tape through a computer-video motion analysis system (Motion Analysis Corp., Model VP 110 and Expertvision software package) interfaced with an Amdek 386 microcomputer. The video sampling rate of the motion analysis hardware was set at 15 frames/s. Analysis of larval behavior consisted of determining swimming speed, net-to-gross displacement ratio (NGDR), rate of change in direction (RCDI), and path duration, for each swimming path. The RCDI is the angle turned per unit time, measured in degree/s. The NGDR is the ratio of the linear distance between the starting and ending points (net distance) and the total distance traversed by the path (gross distance). The NGDR measures the tendency of paths to be circular or twisted and reaches a minimum value of zero for looping or circular paths that have their origin and endpoint at the same spatial coordinates. An NGDR of 1.0 defines a completely straight path.

Data for all paths were pooled across trials for each test or control treatment. The effect of treatments was first assessed using ANOVA. Groups of treatment means were then compared using either *t*-tests or Student-Newman-Keuls multiple range comparisons (SNK). We used an experiment-wise error rate of $\alpha = 0.05$ to determine when comparisons were significantly different.

Initial characterization of settlement inducer

Dose-response curves. Experiments were performed to determine the magnitude of the larval responses to a dilution series of each solution causing settlement. The active solutions were diluted with ASW to 0.31×, 0.1×, 0.031×, and 0.01× their original concentrations. We also tested larval responses to a concentration series of ammonium chloride (pH 8.0), prepared with ASW at 10^{-4} to 3×10^{-7} *M*. Five trials were performed for each solution and concentration. The order of testing was randomized with the constraints described above.

Molecular weight fractionations. Each bath solution that caused settlement was tested both without being fractionated, and after fractionation according to molecular weight. Fractions were prepared by cascading ultrafiltration using a pressure vessel (Amicon Model 8400) and dialysis membranes with cutoffs of <10,000 (Amicon YM10), <1000 (Amicon YM1), and <500 (Amicon YC05) daltons. Ultrafiltrations were performed under a nitrogen atmosphere at 50 psi and 2°C. Five trials were conducted of each solution and ASW, and the order of testing was randomized.

Chemical determinations of metabolite levels

Ammonium and dissolved organic carbon (DOC) were measured to indicate the general metabolite levels of each solution (see Hammen *et al.*, 1966; Srna and Baggaley, 1976; Dame *et al.*, 1989). Ammonium determinations were made with an Alpkem (Model RFA/2) nutrient autoanalyzer, while DOC was measured with a Shimadzu (Model TOC-5000) total organic carbon analyzer.

Methods used in removing biofilms

Microflora were removed from the outer surfaces of cultch and live oyster shells by first vigorously scrubbing with a soft plastic bristle brush to manually dislodge most of the biofilm material. The oysters and cultch then were bathed in 2.5% sodium hypochlorite (NaOCl, Sigma Chemical Co., reagent-grade) for 5 min, which oxidized and further removed microorganisms from the shell surfaces. After the 5 min, each item was removed from the NaOCl and rinsed ten times with 200 ml of sterile ASW. The treated shells were bathed in 1 l of sterile ASW for 5 min, followed by a second set of ten rinses. Living oysters, with shells mechanically and chemically treated, resumed pumping within minutes of being returned to seawater medium.

As a precaution, we performed chemical assays to test that the final rinse waters were free of NaOCl. Rinse waters were analyzed colorimetrically for total chlorine according to the DPD method with N,N-diethyl-p-phenylenediamine reagent. A Hach spectrophotometer (Model DR 2000) was used to measure peak absorbence at 445 nm. Because final rinse waters and ASW both had identical chlorine levels (=2 μg/l), we conclude that the rinses removed all traces of NaOCl.

Microbiological determinations of external biofilms

A series of microbial techniques was used to determine the densities and taxonomic classifications of bacteria found on oyster shells, and to determine the effectiveness of the procedures used to remove biofilms. Three adult oysters and three cultch shells were treated to remove external biofilms through scrubbing and chemical oxidation (see above procedures). These items were then scrubbed for an additional 3 min, with a sterile brush, so that remaining biofilm materials were dislodged and collected in separate beakers each containing 400 ml phosphate buffered saline (PBS). The contents of each beaker were stirred, and four aliquots of 100 ml were then withdrawn. The bacteria in three of the aliquots were enumerated by epifluorescence microscopy, Most Probable Number (MPN) test, and direct plate counts. The numerically dominant bacteria in the fourth aliquot were classified taxonomically.

As a reference for comparison, we repeated the above procedures for an additional three adult oysters and three cultch shells, but with one exception. Shell surfaces were not mechanically and chemically treated initially to remove biofilms. This second group served as a standard by which to determine the effectiveness of scrubbing and chemical oxidation in removing biofilms from the surfaces of shells in the first group, and to identify the dominant bacteria in natural biofilms.

Epifluorescence microscopy. A 5-ml sample of each aliquot was diluted 100-fold with PBS, then 0.01% acridine orange (w/w) was added to stain bacteria cells (Hobbie *et al.*, 1974). We withdrew three 1 ml sub-samples from each of the diluted samples and filtered these separately onto Nucleopore membranes (0.22 μm pore size). The filters containing the stained bacteria were viewed through a fluorescence microscope (Zeiss, Model 47-30-28) and bacteria cells were enumerated according to the procedures of Hobbie *et al.* (1974).

Most Probable Number (MPN). A 10-ml sample of each aliquot was serially diluted ten-fold, 10^1 to 10^5, with PBS. Three, 1 ml sub-samples were then withdrawn from each of the dilutions and added separately to culture tubes, each containing 9 ml alkaline peptone broth. The tubes were incubated for 16 h at 35°C, after which turbidity and MPN estimates of bacteria were determined for each solution.

Direct plate counts. A 1-ml sample of each aliquot was diluted 100-fold with PBS. Three, 100 μl sub-samples were then withdrawn from each of the samples and spread separately on tryptic soy agar plates (TSA, +1% NaCl, Difco Corp.). The plates were incubated for 24 h at 35°C, after which individual colonies were counted.

Identification of dominant bacteria. A 1-ml sample of each aliquot was diluted 100-fold with PBS. Five 100 μl sub-samples of each aliquot were spread separately on replicate TSA plates. The plates were incubated for 24 h at 35°C, after which individual colonies were counted. Bacteria from each colony were then isolated by streaking on separate new TSA plates. These plates were next cultured for 24 h at 35°C. The isolated bacterial colonies were classified to at least the level of Family, by applying

Table III

Mean ammonium [illegible] determined from triplicate assays of bath-waters [illegible] presence of oysters (O), biofilms (B), and shell m[illegible] [illegible]-waters with only clamps (CC) or seawat[illegible] [illegible]WC) were controls

Experiment	Source*	NH_4 (μM)
I	OBS	31.0
	OS	17.5
	BS1	8.7
	S1	0.5
	CC	0.3
	SWC1	0.3
II	BS2	9.1
	S2	0.7
	SWC2	0.4

* The sources of dissolved compounds in these solutions are explained in Tables I and II.

the 23 biochemical analyses of the API 20E system (Analytab, Inc.).

Results

Chemical determinations of metabolites

Concentrations of ammonium in the prepared solutions were between 0.3 and 31.0 μM (Table III), while the dissolved organic carbon concentrations varied from 1.4 to 6.1 mg/l. These values are typical of levels reported for estuarine waters above oyster reefs (Stevens, 1983; Dame *et al.*, 1989; Zimmer-Faust and Tamburri, unpubl. data). Ammonium levels in the OBS, OS, BS1, and BS2 solutions were elevated above concentrations in the seawater control solutions (SWC1 and SWC2), indicating presence of dissolved metabolites, while ammonium levels measured in S1, S2, and CC solutions were nearly identical to the seawater controls, showing an absence of metabolite release. (Abbreviations are fully described in Tables I and II; briefly: O = oyster, B = biofilm, S = shell, and CC and SWC are clamp and seawater controls).

The concentrations of ammonium in the nine test and control solutions (Table III) demonstrate that the techniques used in preparing bath waters resulted in metabolite levels that differed in the expected manner. First, the biofilm removal technique was effective in reducing the amount of metabolites in the OS solution, and in eliminating metabolites in the S1 and S2 solutions. Though still elevated above SWC1, OS contained about half the ammonium as the OBS solution, while ammonium concentrations in S1, S2, SWC1, and SWC2 were essentially identical (all between 0.3 and 0.7 μM). Second, the clamp technique prevented the release of oyster metabolites. This conclusion is supported by our finding that ammonium concentration in BS1 (8.7 μM) was far less than that of the OBS solution (31.0 μM), and nearly identical to the concentration in the BS2 solution (9.1 μM). Third, the techniques of biofilm removal and clamping did not seem to affect metabolite production of the target source, oysters and biofilms, respectively. When ammonium concentrations of OS (17.5 μM) and BS1 (or BS2) solutions are added together, the total concentration of ammonium is similar to the level found in the OBS (31.0 μM) solution.

Microbial determinations

The density of bacteria (cells/cm^2) on external shell surfaces of live oysters and cultch were nearly identical (Table IV). In both cases, 70–75% of the bacteria were in the families Vibrionaceae and Pseudomonadaceae or in the genus *Flavobacteria*. When comparing external oyster shell surfaces, before and after treatment to remove biofilms, most probable numbers (MPN), and direct plate counts reveal a >99% reduction in the total number of viable bacteria (Table IV). The bacterial counts, under epifluorescence microscopy, of treated versus untreated oyster shells show a >90% reduction in bacteria cells. Be-

Table IV

Mean (±SEM) densities of bacteria (No./cm^2) occurring on shell surfaces of living oysters and cultch. Bacteria were counted on shell surfaces with, and without, treatment to remove bacteria biofilms

Experiment and source	Treatment	Most probable number (viable bacteria)	Direct plant count (viable bacteria)	Epifluorescence microscopy (total bacteria)*
I Oysters	Biofilms intact (untreated)	7.9×10^7	$3.1\ (\pm 0.1) \times 10^7$	$6.9\ (\pm 0.7) \times 10^6$
	Biofilms removed (treated)	1.4×10^3	$1.4\ (\pm 0.1) \times 10^3$	$5.9\ (\pm 0.7) \times 10^5$
	Mean % reduction	>99.9	>99.9	91.5
II Cultch	Biofilms intact (untreated)	1.1×10^7	$2.3\ (\pm 0.1) \times 10^7$	$9.1\ (\pm 0.8) \times 10^6$
	Biofilms removed (treated)	9.3×10^3	$1.9\ (\pm 0.1) \times 10^3$	$3.5\ (\pm 0.6) \times 10^5$
	Mean % reduction	>99.9	>99.9	96.2

* Total bacteria includes both living and dead cells.

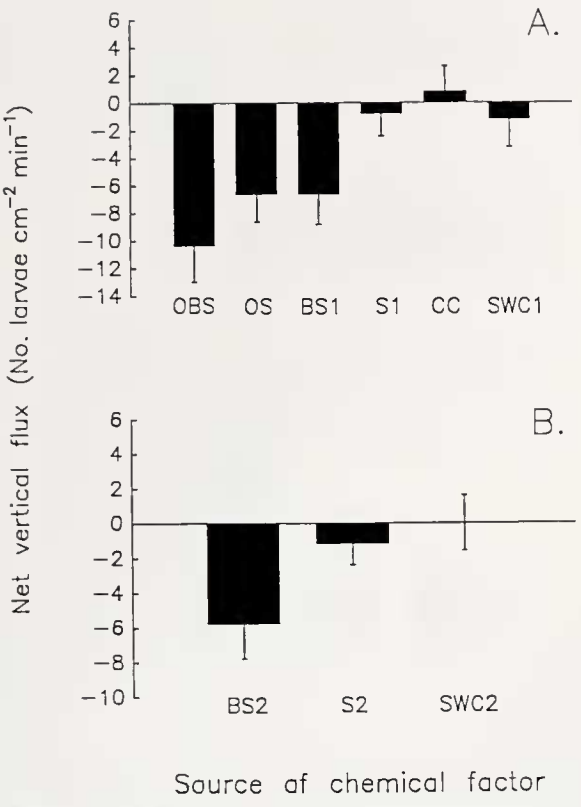

Figure 1A, B. Mean (±SEM) net vertical fluxes of oyster larvae swimming in the water column of the microcosm. Positive values indicate upward transport, while negative values indicate downward transport. The rank order of downward flux in response to solutions is: OBS > OS = BS1 = BS2 > S1 = S2 = CC = SWC1 = SWC2 (Student-Newman-Keuls multiple range comparison: $F = 9.53$; d.f. = 8, 63; $P < 0.0001$). The equal sign (=) designates vertical fluxes that are not significantly different. Abbreviations: oyster (O), biofilm (B), shell material (S), clamp control (CC), and seawater control (SWC).

cause epifluorescence microscopy estimates total bacteria, both living and dead, elevated levels in the treated groups are most likely due to the incorporation of dead cells in the counts.

Larval settlement behavior

The oyster larvae responded to waterborne compounds in the OBS, OS, BS1, and BS2 solutions in a manner indicative of settlement behavior. Larvae swimming in the water column rapidly (within the first 3 min) moved downward when exposed to the OBS, OS, and both BS solutions (Fig. 1). Response to the OBS solution was the largest, showing a significantly greater net downward flux of larvae than to either OS or BS solutions. There was no significant downward movement in response to any other solutions. When exposed to seawater controls (SWC1 and SWC2), larvae exhibited no net vertical movement (−1.00 ± 1.45 SEM and 0.00 ± 1.55 SEM). Larval responses to

Table V

Mean (±SEM) swimming speed of larvae in test and control solutions

Experiment	Solution*	Speed (mm/s)		# of paths analyzed
I	OBS	0.45 ± 0.03	A	71
	OS	0.55 ± 0.04	A	47
	BS1	0.51 ± 0.05	A	41
	S1	0.88 ± 0.05	B	16
	CC	0.93 ± 0.06	B	29
	SWC1	1.03 ± 0.09	B	24
II	BS2	0.51 ± 0.03	A	43
	S2	1.05 ± 0.06	B	25
	SWC2	1.02 ± 0.06	B	18

* Abbreviations: oyster (O), biofilm (B), shell material (S), clamp control (CC), and seawater control (SWC).

Capital letters designate speeds that are not significantly different (Student-Newman-Keuls multiple range comparison: $F = 23.47$; d.f. = 8,305; $P < 0.0001$).

substances released by shells alone (S1 and S2) and clamps (CC), were not significantly different than the responses seen in SWC's.

Larvae also altered their swimming behavior in response to the OBS, OS, and BS solutions, and tended to focus their activity near the bottom. Larvae decreased their swimming speeds (Table V), and they modified their path trajectories as expressed by decreasing NGDRs (Table VI), increasing RCDIs (Table VII), and increasing path durations (Table VIII).

Finally, significantly more larvae attached (with their foot) to the bottom of the microcosm in response to the

Table VI

Mean (±SEM) net-to-gross displacement ratio (NGDR) of larvae swimming in test and control solutions

Experiment	Solution*	NGDR		# of paths analyzed
I	OBS	0.61 ± 0.03	A	71
	OS	0.64 ± 0.03	A	47
	BS1	0.63 ± 0.04	A	41
	S1	0.87 ± 0.05	B	16
	CC	0.85 ± 0.03	B	29
	SWC1	0.82 ± 0.09	B	24
II	BS2	0.62 ± 0.03	A	43
	S2	0.84 ± 0.03	B	25
	SWC2	0.83 ± 0.04	B	18

* Abbreviations: oyster (O), biofilm (B), shell material (S), clamp control (CC), and seawater control (SWC).

Capital letters designate NGDRs that are not significantly different (Student-Newman-Keuls multiple range comparison: $F = 6.91$; d.f. = 8,305; $P < 0.0001$).

Table VII

Mean (±SEM) r[illegible] direction (RCDI) of larvae swimming in test and con[illegible]

Experim[illegible]	[illegible]ution*	RCDI (°/s)		# of paths analyzed
I	OBS	99.77 ± 7.57	A	71
	OS	71.58 ± 6.58	B	47
	BS1	77.19 ± 8.65	B	41
	S1	43.36 ± 7.61	C	16
	CC	58.65 ± 5.85	C	29
	SWC1	51.42 ± 6.20	C	24
II	BS2	65.91 ± 5.15	B	43
	S2	53.83 ± 6.01	C	25
	SWC2	47.52 ± 4.61	C	18

* Abbreviations: oyster (O), biofilm (B), shell material (S), clamp control (CC), and seawater control (SWC).

Capital letters designate RCDIs that are not significantly different (Student-Newman-Keuls multiple range comparison: $F = 6.09$; d.f. = 8,305; $P < 0.0001$).

OBS, OS, and both BS solutions (Fig. 2). The greatest number of settlers were responding to OBS, significantly more than all other solutions. In contrast, when larvae were exposed to S1, S2, and CC solutions, the number of individuals attaching to the substrate was the same as the number settling in the SWC solutions.

In summary, oyster larvae responded to substances in the OBS, OS, and both BS solutions by moving rapidly downward in the water column, by focusing activity near the bottom (by slowing their swimming speed while turning more often), and finally, by increasing their contact and attachment to the substrate.

Initial characterization of settlement inducer

The above behavioral assays indicate that OBS, OS, BS1, and BS2 solutions all contained waterborne settlement factors. Further experiments were conducted with OBS, OS, and BS1 solutions to determine the dose-response relationships, and the molecular weights of settlement inducers. Data are reported only for the number of larvae that attached to the bottom during the observation period, thus simplifying our presentation of results.

Dose-response. Dose-response functions for each solution are expressed as the magnitude of settlement relative to ammonium concentration (Fig. 3). We chose ammonium as the standard, because: (1) it is the principal nitrogen metabolite produced by marine heterotrophic organisms, and (2) it is reported to induce settlement when in the un-ionized form (Coon *et al.*, 1990b). The OBS solution continued to induce significant settlement behavior when diluted 0.031-times its original concentration (0.9 μM ammonium) (Student's t-test: $t = 12.26$, d.f. = 8, $P < 0.001$). Both OS and BS1 solutions were similarly effective when diluted to 0.10-times their original concentrations (0.9 and 1.8 μM ammonium, respectively) (t-test: $t \geq 10.65$, d.f. = 8, $P < 0.001$, both comparisons). In contrast, larvae did not settle at rates significantly higher than seawater control (SWC1) in response to any test concentration of ammonium chloride.

Each dose-response curve was analyzed according to a non-linear regression model, assuming a hyperbolic relationship between concentration of the ammonium standard and the magnitude of settlement response (Statistical Analysis Systems, Carey, North Carolina, SAS version 6; also see theoretical arguments of Beidler, 1954; Maes, 1984). The curves generated for OBS, OS, and BS1 solutions all produced highly significant fits to the model (F-test: $F \geq 28.22$; d.f. = 2, 17; $P < 0.001$, all comparisons). Asymptotes and slopes of curves generated in response to OS and BS1 solutions were identical (t-tests: $P > 0.50$, both comparisons). However, there was a significant difference in the asymptotes between the dose-response curves describing settlement in response to OBS and either OS or BS1 solutions (t-tests: $P < 0.01$, both comparisons), but not in the slopes (t-test: $P > 0.05$, both comparisons).

Molecular weight fractionations. Compared with the seawater control (SWC1) larval settlement was significantly elevated in the <10,000 and <1000 molecular-weight fractions of OBS, OS, and BS1 solutions (SNK: critical range ≥ 1.201, $P < 0.01$) (Fig. 4). Levels of response were the same for these fractions and for solutions tested without fractionation (SNK: critical range ≥ 1.197, $P > 0.20$). The larvae did not settle at levels elevated from SWC1, when exposed to the <500 molecular-weight frac-

Table VIII

Mean (±SEM) path duration of larvae swimming in test and control solutions

Experiment	Solution*	Duration (s)		# of paths analyzed
I	OBS	8.45 ± 1.19	A	71
	OS	6.91 ± 0.65	B	47
	BS1	7.26 ± 0.80	B	41
	S1	4.19 ± 0.50	C	16
	CC	3.85 ± 0.31	C	29
	SWC1	4.05 ± 0.36	C	24
II	BS2	8.39 ± 0.89	A	43
	S2	4.20 ± 0.44	C	25
	SWC2	4.18 ± 0.48	C	18

* Abbreviations: oyster (O), biofilm (B), shell material (S), clamp control (CC), and seawater control (SWC).

Capital letters designate path durations that are not significantly different (Student-Newman-Keuls multiple range comparison: $F = 20.73$; d.f. = 8,305; $P < 0.0001$).

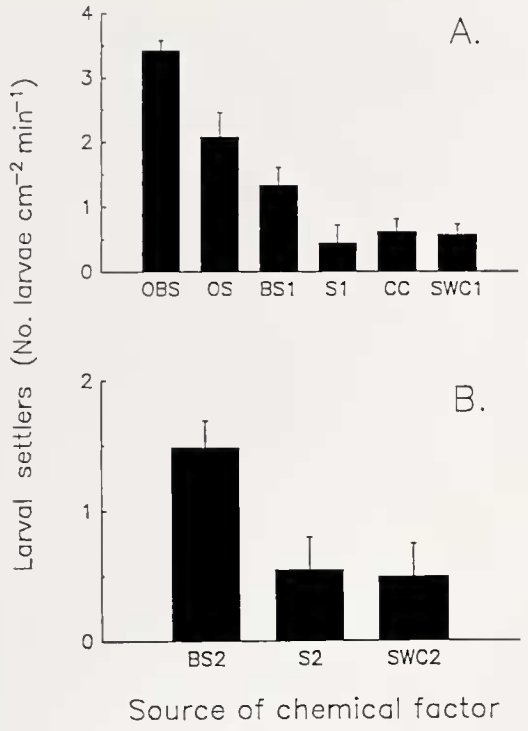

Figure 2A, B. Mean (±SEM) settlement rates of oyster larvae. The ranked order of settlement in response to solutions is: OBS > OS = BS1 = BS2 > S1 = S2 = SWC1 = SWC2 (Student-Newman-Keuls multiple range comparison: $F = 20.43$; d.f. = 8, 63; $P < 0.0001$). The equal sign (=) designates settlement rates which are not significantly different. Abbreviations: oyster (O), biofilm (B), shell material (S), clamp control (CC), and seawater control (SWC).

tions (SNK: critical range ≥ 0.981, $P > 0.20$). Consequently, the data indicate that the settlement inducers in each solution (OBS, OS, and BS1) have molecular weights between 500 and 1000 daltons.

Discussion

Ever since Cole and Knight-Jones (1939) first described the gregarious nature of larval settlement in oysters, there has been debate about the sources of chemical inducers. Two schools of thought have emerged: one reports juvenile and adult oysters to be the source of inducers, whereas the other finds the source in biofilms (references in Introduction). Our results suggest that these contrasting viewpoints have resulted largely from differences in experimental approach. In this study we have separated both proposed sources of settlement inducers by preparing and testing solutions that contain compounds released either by oyster or by their external biofilms. We report here that *both* sources produce waterborne substances rapidly provoking larval settlement.

The small size of oyster larvae (250–350 μm), and their sensitivity to handling, made them difficult subjects for

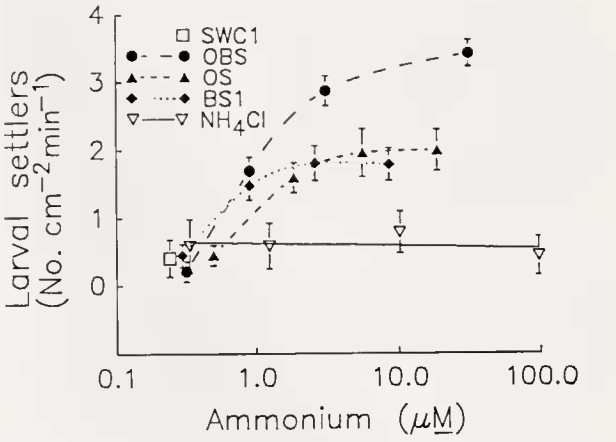

Figure 3. Dose-responses of oyster larvae to serial dilutions of OBS, OS, BS1, and ammonium chloride (NH_4Cl) solutions, relative to seawater control (SWC1). Responses are expressed as mean (±SEM) settlement rates; dosages are expressed in ammonium concentrations. Abbreviations: oyster (O), biofilm (B), and shell material (S).

experimental analysis. We overcame these difficulties by applying computer-video motion analysis to non-invasively track the paths made by individual larvae as they swam in the water column or crawled on the substrate. We found this technology to be indispensable to the study of larval behavior, because: (1) experimental chambers having large volumes (*e.g.*, 30–5000 ml; Zimmer-Faust, 1990; Weissburg and Zimmer-Faust, 1991; present study) can be used, which do not impede locomotory behavior, (2) locomotory paths can be tracked for many individual larvae simultaneously, and (3) data can be collected rapidly and in sufficient quantities from larvae held at low densities (1 or 2/ml), thereby avoiding interactions between larvas and density-dependent effects.

We isolated the oyster metabolites (from biofilm metabolites) during the preparation of the OS solution by

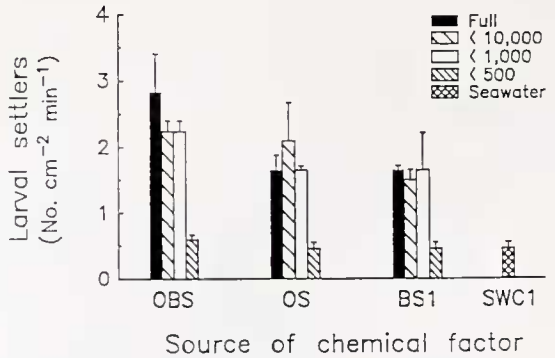

Figure 4. Mean (±SEM) settlement rates of larvae in response to seawater control (SWC1), and to OBS, OS, and BS1 solutions tested without fractionation (Full), and after fractionation by molecular weight to <10,000, <1000, and <500 daltons. Abbreviations: oyster (O), biofilm (B), and shell material (S).

minimizing the amount of biofilm organisms on the outer shell surfaces. Our efforts were focused on eliminating external biofilm because bacteria in oyster tissues, on internal shell surfaces, and released during incubations of oysters were numerically insignificant (≤0.1%) compared with the total on external shell surfaces. Not only was there a >99% reduction in total viable bacteria after treatment to remove external biofilms, but there was also a large reduction in ammonium levels in the OS solution relative to the OBS solution. Significantly more larvae settled in response to the OS solution than in response to SWC1, but the reduction of metabolites in the OS solution coincided with a significant (≈2-fold) decrease in larval settlement response when compared to the OBS solution.

Biofilm metabolites were collected for assay by minimizing, or eliminating, the influence of live oysters in preparing the BS solutions. Both solutions (BS1 and BS2) induced significant settlement activity, although not to the same extent as the OBS solution. The reduction in settlement activity in BS1 and BS2 corresponded to a reduction in ammonium and DOC levels (relative to the OBS), yet the concentrations were almost identical in BS1 and BS2. These results indicate: (1) that the clamping technique is effective in preventing the oysters from releasing significant levels of metabolites in the BS1 solution, and (2) clamped oysters (BS1) and shell cultch with intact biofilms (BS2) both release substances evoking settlement. This second point clearly demonstrates that biofilms are producers of chemicals inducing oyster larval settlement.

The potential influence of the shell material itself was never completely isolated from oyster and biofilm (OBS, OS, BS1, and BS2). Nevertheless, both solutions prepared with shells alone (S1 and S2) had ammonium concentrations equal to seawater controls (SWC1 and SWC2). Because neither S1 nor S2 induced larvae to settle, the effect of shell material was negligible. We admit the technique used to remove biofilms was imperfect, because some cracks or crevices harboring pockets of viable bacteria probably went undetected. Still, the absence of settlement responses by larvae to S1 and S2 argues that sufficient amounts of bacterial biofilms were removed, so if inducers were produced, their levels were below the threshold for settlement.

Larvae responded similarly to oyster and biofilm metabolites by swimming actively downward to the bottom of the test chambers. Once near the bottom (within four larval body lengths), they decreased their swimming speed and net-to-gross displacement (NGDR), while they increasing their rate of turning (RCDI) in the horizontal plane. These changes in swimming near the bottom are consistent with site-restricted search (*e.g.*, see references on zooplankton by Buskey, 1984; Buskey and Stoecker, 1989; Weissburg and Zimmer-Faust, 1991). Clearly, increased turning may facilitate 'search' for specific microsites, because turning causes larvae to loop back focusing locomotory activity within a small area (*e.g.*, Koopman, 1980). The longer mean durations of paths recorded as larvae swam near the bottom in response to OBS, OS, BS1, and BS2 support this conclusion (Table VIII). The larvae tended to concentrate their swimming activities within an area the size of our viewing field (6.75 mm × 6.70 mm), or smaller. Larvae also attached with their foot and crawled on the bottom, clearly indicating a settlement response.

We have previously assayed behavior of larvae produced by Chesapeake Bay and Galveston Bay oysters. Oyster populations in these two regions are of different physiological strains (Groue and Lester, 1982; Buroker, 1983; King and Gray, 1989). Yet, the responses to oyster metabolites exhibited by larvae of both populations were the same (Zimmer-Faust and Tamburri, in prep.). Our current investigation shows that larvae behave in identical fashion to chemical factors released independently by adult oysters and by biofilms. Consequently, the sequence of behaviors in settlement is genetically conserved and appears stereotyped in response to chemical stimuli produced by both sources.

The molecular structures of compounds inducing settlement remain unresolved. Soluble bacterial metabolites ≤ 300 daltons, and ammonia, are proposed to elicit settlement responses (Fitt *et al.*, 1989, 1990; Coon *et al.*, 1990b). Our data seem to be in contrast with this hypothesis, because in each case fractions prepared at <500 daltons were ineffective as settlement stimuli. In fact, solutions of ammonium chloride did not induce settlement responses at concentrations tested in this study. The threshold for settlement induction by ammonium chloride (at pH 8.0) is 2.5 m*M* (Coon *et al.*, 1990b), a concentration 100-times higher than those in OBS, OS, and BS1 solutions maximally stimulatory to settlement in our dose-response experiments.

Additional investigation is needed to further identify the structures of settlement inducers. The narrow range of molecular weights of compounds stimulatory to settlement (500–1000 daltons), and the marked similarity between dose response curves for OS and BS1 solutions, argue for production of the same factors by both oysters and biofilms. Alternatively, we found the asymptote of the dose—response curve for the combined source (OBS) to be significantly higher than that for the dose—response to either single source, OS, and BS1. This difference between asymptotes suggests that each source might produce distinct inducers, differing in structure, yet acting together synergistically when combined in mixtures.

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